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Protein Storage Bodies and Vacuoles

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INTRODUCTION

Plants store proteins in embryo and vegetative cells to provide carbon, nitrogen, and sulfur resources for subsequent growth and development. The storage and mobilization cycles of amino acids that compose these proteins are critical to the life cycle of plants. Mechanisms for protein storage and mobilization serve many different developmental and physiological functions. For example, stored protein provides building blocks for rapid growth upon seed and pollen germination. Similarly, protein reserves in vegetative cells provide the building blocks for seed and fruit set during reproductive growth and for rapid expansion of vegetative structures after periods of dormancy. In agriculture, proteins stored in seeds and vegetative tissues account for much of the protein consumed directly as food by humans and livestock. Consequently, the biochemistry of storage proteins and the cellular and physiological mechanisms regulating their synthesis are of practical as well as academic interest.

In this brief review, we discuss the nature of protein storage bodies and the cellular processes involved in the accumulation of storage proteins. Storage proteins accumulate primarily in the protein storage vacuoles (PSVs) of terminally differentiated cells of the embryo and endosperm and as protein bodies (PBs) directly assembled within the endoplasmic reticulum (ER). The synthesis of storage proteins and the formation of specialized vacuoles occur after cell division is complete, when all further growth occurs only through cell expansion and accumulation of storage substances. In the past, the terms PB and PSV have been used interchangeably, but PSV is now used to differentiate vacuoles containing storage proteins from PBs originating from the ER. Our understanding of the cellular context in which storage proteins accumulate derives from many significant advances in gene structure and regulation, as well as the biochemistry and morphogenesis of storage tissues (reviewed in Chrispeels, 1991; Thomas, 1993; Staswick, 1994; Shewry et al., 1995; Galili and Herman, 1997; Nielsen et al., 1997; Shewry and Tatham, 1998; see also Battey et al., 1999; Marty, 1999; Sanderfoot and Raikhel, 1999; Vitale and Denecke, 1999, in this issue).

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STORAGE PROTEINS ARE ASSEMBLED IN THE ER

PBs form as a consequence of developmentally regulated events that induce storage protein synthesis in specialized cells and promote storage protein accumulation in specific organelles. All storage proteins are initially synthesized on the rough ER (Bollini and Chrispeels, 1979; reviewed in Chrispeels, 1991). This membrane system consists of an extensive, interconnected network of tubules and cisternae (reviewed in Staehelin, 1997) and serves as the port of entry for secretory and membrane proteins. Storage proteins may remain in the ER or be transported through the endomembrane system to distal sites (Figure 1; reviewed in Vitale and Denecke, 1999, in this issue). The initial synthesis of storage proteins may be restricted to specific subdomains of the ER; however, this question has not yet been thoroughly investigated.

The entry of storage proteins into the ER occurs cotranslationally and is specified by an N-terminal signal peptide that is cleaved from the nascent polypeptide chain as it enters lumenal space (Von Heijn, 1984). Further processing of storage proteins within the ER appears to include their folding and oligomerization, processes facilitated by lumenal chaperones and enzymes (reviewed in Boston et al., 1996; Vitale and Denecke, 1999, in this issue). Mutations of a storage protein in maize, for example, can induce the synthesis of the molecular chaperone binding protein (BiP) as well as protein disulfide isomerase (Zhang and Boston, 1992; Li and Larkins, 1996). It has furthermore been established that the formation of oligomers of 11S proglobulin (a legumin-type globulin) storage proteins in vitro requires both ATP and molecular chaperones (Nam et al., 1997). In addition, members of several storage protein families, including the seed 7S (vicilin-type globulin) as well as vegetative storage protein (VSP), are cotranslationally glycosylated within the ER (reviewed in Bollini et al., 1983; Faye et al., 1989; Chrispeels, 1991).

The major seed vacuolar storage proteins, (i.e., 7S and 11S globulins), as well as many ancillary storage proteins, form dimers, trimers, and tetramers in the ER lumen shortly after synthesis (Chrispeels et al., 1982a, 1982b; Ceriotti et al., 1995). Mutant proteins that are unable to form the correct quaternary structures are retained and degraded in the ER (Vitale and Denecke, 1999, in this issue). Oligomers of the 7S and 11S globulins are soluble and are transported to vacuoles by progression through the endomembrane system, where they form PSVs. In contrast, the prolamin storage proteins of cereals form large oligomeric aggregates in the ER. In maize and rice, these protein accretions are retained within the ER (Larkins and Hurkman, 1978; Li et al., 1993a, 1993b), whereas in wheat, the protein accretions are budded from the ER in the form of PBs encased in an ER-derived membrane (Figures 1C and 1D). These PBs can become sequestered into provacuoles that eventually fuse, forming one or more large central vacuoles that contain numerous protein accretions (Levanony et al., 1992; Galili et al., 1993; see Marty, 1999, in this issue). The assembly of prolamins to form PBs within the ER undoubtedly reflects the unusual structures of these proteins (reviewed in Shewry and Tatham, 1998).

All prolamins are soluble in aqueous alcohol solutions, which reflects their general hydrophobic nature. However, there is significant variation in the primary structures of prolamins from different groups of cereals such as Triticeae (wheat, barley, and rye) and the Panicoideae (maize, sorghum, and millet). Prolamins of both groups contain sulfur-poor and sulfur-rich types and possess a high percentage (30 to 70%) of proline and glutamine, hence the name prolamin. Prolamins thus appear to have evolved through amplification of proline- and glutamine-rich peptides, containing from three to 20 amino acids. In some cases, these repeated, hydrophobic sequences account for the majority of the protein.

In light of their hydrophobicity, it might be presumed that prolamins aggregate in a nonspecific manner within the lumen of the ER. If the aggregation of prolamins into protein bodies were simply determined by hydrophobic interactions, the ER would become filled with protein, much like a sausage casing. Instead, results from several recent studies in maize, barley, and wheat suggest a model in which prolamins are organized into PBs by specific interactions between sulfur-rich and sulfur-poor prolamins (Rechinger et al., 1993; Coleman et al., 1996; Bagga et al., 1997). Furthermore, targeting of prolamin mRNAs to the ER may play an important role in directing these proteins to specific domains of the membrane, where they form oligomers (Li et al., 1993a).

PBs in maize endosperm form directly in the lumen of the rough ER and contain at least four distinct prolamins—the α-, β-, γ-, and δ-zeins (Larkins et al., 1989). The PBs of smallest diameter apparently contain β- and γ-zeins, which are cysteine-rich and cross-linked by disulfide bonds (Lending and Larkins, 1989; Lopes and Larkins, 1991). The α- and δ-zeins penetrate the matrix of β- and γ-zeins and expand the PB into a larger spherical structure that reaches a diameter of 1 to 2 μm.

The mechanisms that limit the further expansion of the protein body are unknown, but it appears that γ-zein and perhaps β-zein organize the α- and δ-zeins and retain them within the ER lumen. Specifically, when β- or γ-zeins are synthesized in transgenic tobacco leaves or endosperm, they are retained within the ER (Coleman et al., 1996; Bagga et al., 1997). When α- or δ-zeins are synthesized in these tissues, however, both proteins appear to be secreted and become degraded (Williamson et al., 1988; Coleman et al., 1996; Bagga et al., 1997). α- or δ-zeins will accumulate into complexes, however, when coexpressed with β- and γ-zeins in transgenic tobacco. This suggests that β- and γ-zeins provide the ER retention mechanism for α- and δ-zeins. Consequently, the amount of β- and γ-zein in a PB may limit its growth.

The nature of physical interactions between zein proteins that are responsible for PB assembly are unknown; however, deletion mutants of γ-zein show that proline-rich repeats at the N terminus of the wild-type protein direct its retention within the ER (Geli et al., 1994). A mutant α-zein protein with a nonfunctional signal peptide (Coleman et al., 1997) causes the β- and γ-zeins to become displaced from the periphery of the PB, resulting in PBs that bud and grow irregularly as the mutant α-zein accumulates (Lending and Larkins, 1992; Zhang and Boston, 1992). This result is also consistent with a model in which the role of the β- and γ-zeins is to organize and partition the more hydrophobic α- and δ-zeins in the center of the protein body.

Prolamin assembly into PBs is a no less complex process in wheat and barley. Whereas prolamin accretions in these cereals also form within the ER lumen, they are subsequently transported to PSVs by either of two different routes (Galili et al., 1993; see below). In barley, the various types of sulfur-rich and sulfur-poor prolamins tend to partition into distinct PBs within the ER (Rechinger et al., 1993), whereas in wheat, there is more integration of the sulfur-rich and sulfur-poor prolamins within PBs (Rubin et al., 1992).

The complexity of the post-translational processing of wheat and barley prolamins appears to be related to their intricate structures. For example, Altschuler et al. (1993) demonstrated that a sulfur-rich wheat γ-gliadin contains a series of N-terminal tandemly repeated peptides that cause it to be retained within the ER and an autonomous C-terminal region that targets it for secretion. Thus, the effective trafficking of these proteins to the ER or Golgi complex may be determined by the relative strength of the targeting signals within the various prolamin types. The interactions between sulfur-rich and sulfur-poor prolamins in wheat and barley have not been thoroughly investigated, but these associations could be important in subcellular targeting, as evidenced by ultrastrucural studies of barley mutants deficient in the sulfur-rich γ-hordeins. The variety Nevsky lacks γ3-hordein, a unique monomeric protein that forms intramolecular disulfide

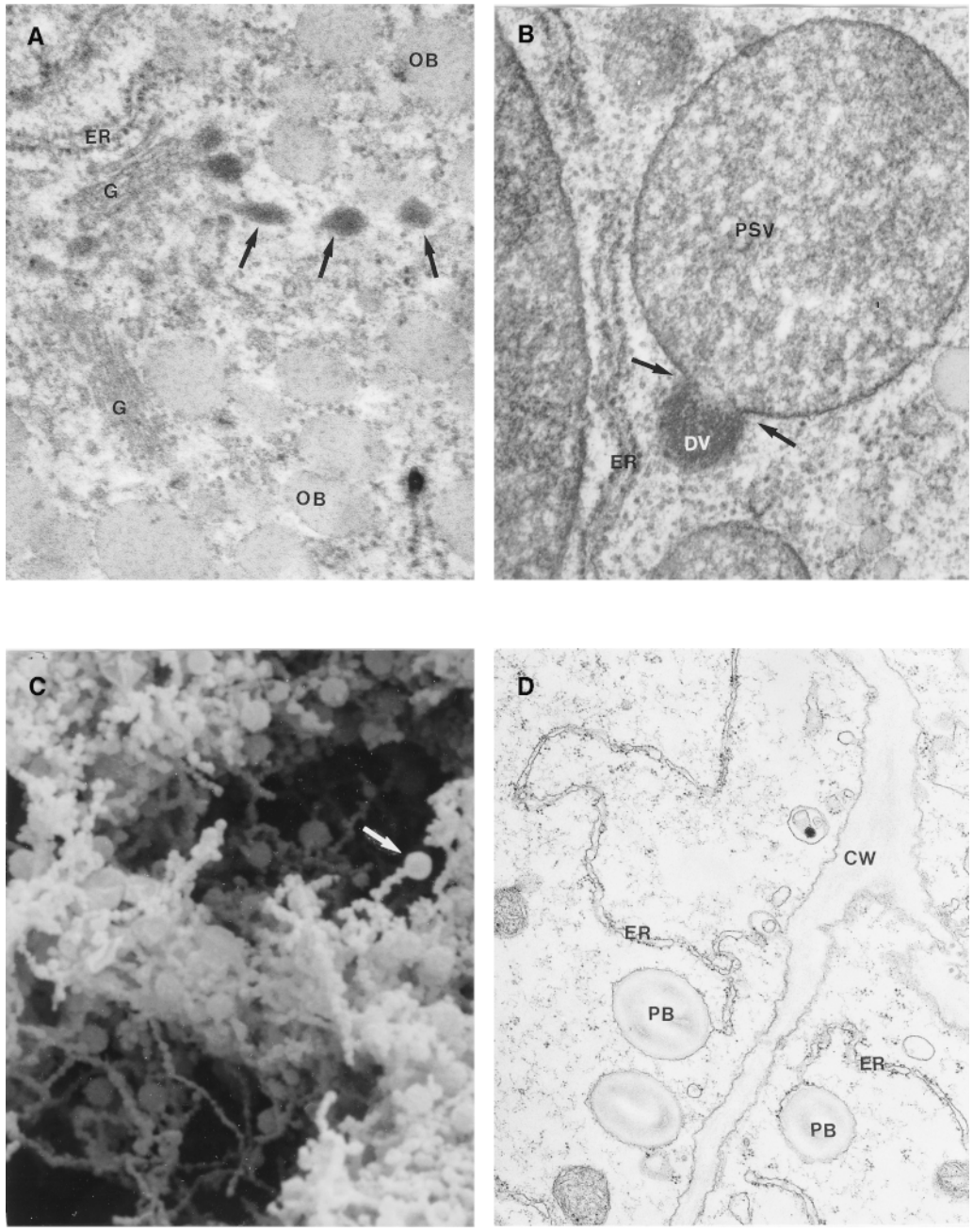

Figure 1. Ontogeny of Organelles Involved in Protein Storage in Seeds.

(A) Conventional electron microscopy of a midmaturation soybean seed storage parenchyma cell showing the Golgi complex (G) secreting dense vesicles (arrows) that sequester storage protein precursors. OB, oil body.
(B) The apparent fusion (arrows) of a dense vesicle (DV) to the protein storage vacuole (PSV) in a midmaturation soybean parenchyma cell.
(C) Scanning electron microscopy of a developing maize endosperm cell. The extensive system of tubular rough ER is observed, with protein bodies occurring at the ends of the ER membranes (arrow).
(D) Electron micrograph of maize endosperm at 19 days after pollination. The rough ER is discontinuous in cross-section but continuous in surface section. The ER is connected to protein bodies (PB). CW, cell wall.

bridges. In endosperm of this genotype, the sulfur-poor β-hordeins accumulate in the ER, rather than being transported to the vacuole. ER retention of B-hordein is not observed in mutants lacking γ1- or γ2-hordein, which form intermolecular disulfide bridges (Rechinger et al., 1993). Consequently, it appears that the γ3-hordein contributes to the transport competence of some of the sulfur-poor hordeins.

The organization of prolamins into protein accretions may not simply be determined by protein–protein interactions within the lumen of the ER. It is possible that prolamins are targeted to specific regions or subdomains of the ER via their mRNA sequences. Mechanisms for targeting mRNAs are well known in animal cells, where this process has been shown to be mediated by the cytoskeleton and proteins that interact (most frequently) with the 3′ noncoding sequence of mRNAs (St. Johnston, 1995). This device provides an effective way for soluble and secreted proteins to be synthesized proximal to the point along the secretory pathway at which they are needed, and it also provides a mechanism to separate proteins that might otherwise interact inappropriately (Rings et al., 1994).

Although mRNA sorting has not been widely investigated in plants, Okita and co-workers have found evidence for this phenomenon in developing rice endosperm (Li et al., 1993a). Rice contains two types of storage proteins: prolamins, which form accretions within the lumen of the ER; and glutelins (related to 11S globulins), which are synthesized on rough ER and then transported to PSVs. Li et al. (1993a) found that whereas both types of mRNAs are found in rough ER polysomes, the prolamin transcripts are preferentially localized on membranes surrounding prolamin-containing protein bodies, and glutelin mRNAs are predominantly associated with polysomes on the cisternal ER. Although the mechanism responsible for the asymmetric distribution of the two types of mRNAs is unknown, this observation suggests a process by which prolamin mRNAs could simultaneously direct the synthesis and organization of prolamins into protein bodies.

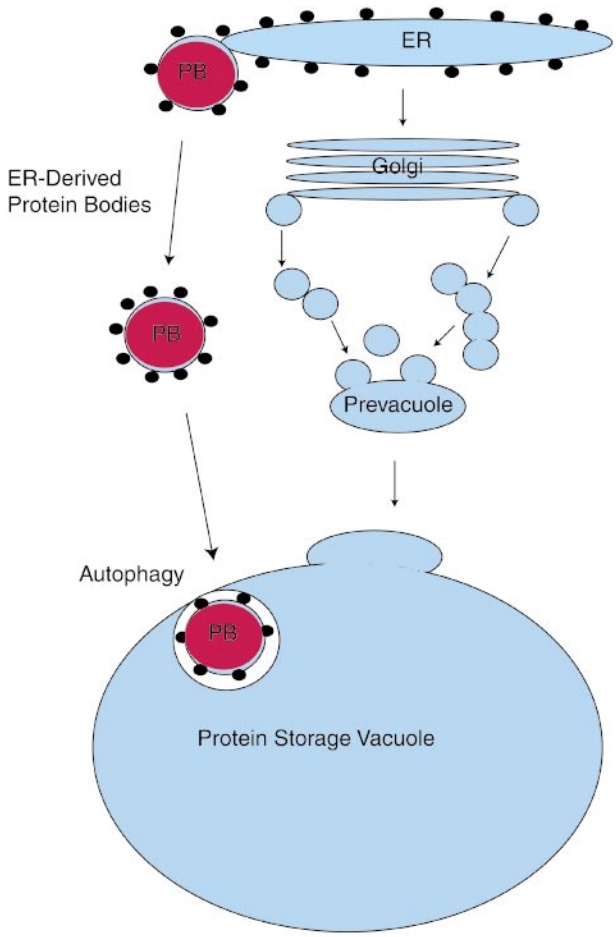

Figure 2. Conceptual Diagram of the Ontogeny of PBs and PSVs.

PBs are assembled through the aggregation of proteins within the ER. After formation, they can either remain attached to the ER or bud off as separate organelles. PBs can then either accumulate in the cytoplasm or be sequestered into vacuoles by autophagy. PSVs are formed as the consequence of ER-synthesized storage proteins progressing through the endomembrane secretory system to the vacuole for accumulation.

TRAFFICKING OF STORAGE PROTEINS FROM THE ER TO THE PSV

We have discussed how prolamins in maize and rice are retained within ER-derived PBs (Larkins and Hurkman, 1978; Li et al., 1993b). In other cereals, such as wheat, PBs can be sequestered into one or more large central vacuoles that contain numerous PBs (Galili et al., 1993). We shall now discuss the two alternative routes that PBs can take for vacuolar sequestration (Figure 2). One route involves protein transport through the Golgi complex, a process that is discussed extensively elsewhere in this issue (Sanderfoot and Raikhel, 1999). The second route may be particular to storage proteins and occurs by autophagy.

Autophagy is the primary route by which plant cells dispose of cytoplasmic constituents and materials internalized from the extracellular space by multivesicular endosomes (reviewed in Herman, 1994; Robinson et al., 1998). In some cereals, autophagy is also used to accumulate storage proteins, bypassing the conserved mechanism of Golgi-mediated targeting and transport to the vacuole (Levanony et al., 1992). Storage proteins assembled in the ER are polymerized into higher order structures (Shimoni and Galili, 1996) that are directly secreted from the ER. The wheat PBs do not remain as separate cytosolic structures but are instead sequestered into provacuoles (Rubin et al., 1992). The origin of the provacuolar membranes—whether from the Golgi

complex or directly from the ER—has not been determined; however, these membranes are known to carry the tonoplast marker proteins γ-TIP (for tonoplast integral protein) and pyrophosphatase, both of which are characteristic of vacuole formation mediated by the Golgi complex (G. Galili, personal communication). The provacuoles containing sequestered PBs fuse one to another, forming one or more large central vacuoles that contain numerous storage protein aggregates. The limiting membrane of the sequestered PB appears to be digested by vacuolar enzymes, releasing the naked prolamin aggregate into the vacuolar sap. The prolamin protein accretions subsequently aggregate, forming larger PBs.

Autophagy of prolamin-containing protein bodies (Figure 2) can be observed in transgenic plants. Coleman et al. (1996) established that coexpression of α- and γ-zein in tobacco seeds resulted in the production of PBs that appear to be structurally indistinguishable from maize PBs. The cytosolic PBs possess a limiting membrane with bound ribosomes that sequesters a protein matrix of γ-zein with included loculles of α-zein. Electron microscope immunocytochemistry indicated that PBs become sequestered within PSVs by autophagy. Quantitative differences in γ- and α-zein content of the cytoplasmic and PSV-sequestered protein bodies indicate that α-zein is unstable once the PB is taken into the vacuole. As discussed above, Bagga et al. (1995, 1997) described similar results for β/δ-zein–containing PBs formed in transgenic tobacco seeds and leaves. Taken together, these results indicate that the autophagic process that occurs in wheat endosperm, whereby ER-derived PBs are sequestered in vacuoles, can be duplicated by producing PBs in transgenic tobacco, even though tobacco plants do not normally produce ER-derived PBs.

There are recent indications that 11S storage proteins that would normally be transported to the vacuole by endomembrane progression can be sequestered in ER-derived PBs. Hara-Nishimura et al. (1998) have shown that maturing pumpkin cotyledon cells contain ER-derived PBs that sequester the precursor of the 11S storage protein. Similarly, A.J. Kinney and E.M. Herman (unpublished data) have found that cosuppression of the 7S storage protein, conglycinin, in transgenic soybeans promotes the accumulation of PBs that contain the 11S storage protein, glycinin. These results indicate that PBs can sequester vacuolar proteins diverted from endomembrane progression in addition to the well-characterized role of the PB in sequestering prolamin storage proteins.

TRANSPORT OF STORAGE PROTEINS VIA THE ENDOMEMBRANE SYSTEM

Transit of Proteins from the ER to the Golgi Complex

The residence of storage proteins within the ER is characterized by a half-life of several hours (Chrispeels et al., 1982a), after which they may transit through the Golgi complex. Conventional electron micrographs of proteinaceous electron-dense vesicles exiting the *trans* cisternae of the Golgi complex and immunogold techniques have confirmed that storage proteins transit the ER and the Golgi complex before entering secretory vesicles that form PSVs (Figures 1A and 1B; Craig and Goodchild, 1984; Herman and Shannon, 1984a, 1984b; Greenwood and Chrispeels, 1985). In addition to its role in protein transport and packaging, the Golgi complex may process the ER-derived high-mannose glycan side chains of vacuolar glycoproteins (Chrispeels, 1983; Faye et al., 1989; reviewed in Staehelin and Moore, 1995). The acquisition of xylosyl residues on a storage protein of soybean aleurone cells, also visualized by immunogold electron microscopy, further indicates the role of the medial to *trans* domain of the Golgi complex in processing storage proteins prior to their arrival in the PSV (Yaklich and Herman, 1995). Glycosylation of the major seed PSV proteins is predominantly N linked, although the sweet potato tuber vacuolar storage protein sporamine possesses O-linked glycan side chains (Matsuoka et al., 1995). Because glycans do not appear to have a role in vacuolar targeting (Sturm et al., 1988), the significance of the glycosylation of storage proteins remains unclear.

Transit of Storage Proteins from the Golgi Complex

Protein targeting to the vacuole appears to occur in the Golgi complex. Peptide targeting sequences have been identified and/or inferred for many storage vacuole constituents, including enzymes and storage proteins (reviewed in Chrispeels and Raikhel, 1992; Sanderfoot and Raikhel, 1999, in this issue). There appear to be several different types of unrelated sequences that target multiple receptors. The best-characterized receptors are BP-80 and its homologs that recognize N-terminal prodomain sequences found on vacuolar cysteine proteases (Holwerda et al., 1992) and a few other proteins (Ahmed et al., 1997; Paris et al., 1997).

Other targeting sequences occur at the C-terminal prodomain of the wheat germ agglutinin family of lectins and the closely related chitinases (Wilkins et al., 1990; Neuhaus et al., 1991). The receptor for these C-terminal targeting sequences has not been identified. The targeting sequences of the major storage proteins, including the 11S and 7S globulin families as well as the seed lectins, also have not been identified. Paris et al. (1997) showed that the BP-80 receptor appears to be associated with the *trans*-Golgi network (TGN) and its derived secretory vesicles, and they postulated that the receptor may become detached from secretory vesicles during transit to the vacuole. Thereafter, the receptor may recycle back to the TGN, as do vacuole protein receptors in yeast cells (Cooper and Stevens, 1996).

Although prolamin proteins in wheat and barley do not appear to possess vacuole-targeting sequences, they could

nevertheless progress to the vacuole via the Golgi complex. Wheat prolamins are in fact detectable in the Golgi complex (Krishnan et al., 1986). The addition of the ER retention tetrapeptide sequence KDEL to a wheat gliadin, expressed in transgenic tobacco, stabilized the protein, suggesting that the site of degradation is post-*cis* Golgi and that gliadins progress through the endomembrane system (Napier et al. 1997).

Storage proteins exit the TGN packaged in electron-dense vesicles of ~0.1 μm diameter (Figures 1A and 2). The dense vesicles contain Golgi-processed, precursor storage proteins (Chrispeels, 1983). Storage proteins do not appear to enter the TGN, which remains unlabeled in immunodetection assays. Immunological methods (Harley and Beevers, 1989) suggest that clathrin-coated vesicles mediate some trafficking to the vacuole. This hypothesis is difficult to reconcile with electron microscopic observations that show storage proteins leaving the Golgi complex packaged in dense vesicles. Furthermore, a recent study provides compelling data that refute a role for coated vesicles in storage protein transport (Hohl et al., 1996).

Maturing cotyledon cells contain dense vesicles 0.3 to 0.5 μm in diameter that carry storage proteins and appear to be identical to secretion vesicles (see, e.g., Herman and Shannon, 1985; Hara-Nishimura et al., 1993b, 1995; Robinson et al., 1997; for additional discussion, see Robinson et al., 1998; Figure 2). The superficial appearance of these larger dense vesicles argues that they arise from the 0.1-μm Golgi secretion vesicles, although several 0.1-μm Golgi secretion vesicles would need to aggregate to account for the volume and membrane surface of the larger dense vesicles. The question of how 0.3- to 0.5-μm dense vesicles originate from 0.1-μm Golgi secretion vesicles may be addressed by a recent observation of Hirschberg et al. (1997). By using a green fluorescent protein fusion to a secretory protein, they followed the path of synthesis and lysosomal transport of proteins to and through the Golgi complex in animal cells. Time-course video-enhanced fluorescence light microscopy showed that the initial Golgi secretion event does not involve individual vesicles but rather an undulating tube of vesicles, appearing as linked beads that extend outward from the Golgi complex along cytoskeletal elements before collapsing into a larger vesicle. Reexamination of published seed Golgi secretion vesicles in photographs of both freeze-fracture and thin-sectioned material similarly shows elongated vacuoles that may be analogous to the tubules observed by Hirschberg et al. (1997).

The 0.3- to 0.5-μm dense vesicles may also be analogous to the 1.0-μm diameter small protein-filled vesicles in root tips and aleurone cells that have recently been termed second vacuoles (Paris et al., 1996). These second vacuoles contain newly synthesized proteins and possess tonoplasts with different polypeptides than do preexisting vacuoles. Second vacuoles have been observed in root tips and in barley aleurone cells, where they are also termed aleurain (cysteine protease)-containing vacuoles. If the analogy to seed cells proves to be correct, then the second vacuoles would constitute a transient population of prevacuolar compartments.

PSVs originate from post-Golgi central vacuoles that are devoid of significant protein accumulation in both embryo and vegetative cells (Figure 2). Storage proteins are added and gradually fill the vacuole. This is in contrast to the ER-derived PBs, which form as protein accretions and do not undergo further alteration. PSVs are structurally differentiated as transient subdivisions of the preexisting vacuole, and this occurs coordinately with the onset of storage protein synthesis and accumulation. Craig et al. (1980) used light microscopy to follow the process of subdivision of the vegetative vacuole of pea cotyledon cells. Their studies and those of several other groups show that this results in up to a 1000-fold increase in the total membrane area as the single vegetative vacuole is subdivided into numerous PSVs (Craig et al., 1979, 1980). Not all investigators agree that the ontogeny of the PSV is the result of subdivision of a preexisting vacuole. Robinson et al. (1995) assert that the vacuole that is transformed into a PSV is synthesized de novo and replaces the preexisting vegetative vacuole. According to this hypothesis, it is this new vacuole that is subdivided into the numerous PSVs.

PROTEIN STORAGE WITHIN THE PSV

Protein Storage in Seeds

The presence of storage proteins defines the seed PSV. Mature seeds contain densely packed storage protein deposits that entirely fill the PSV. PSV protein deposits may be uniformly amorphous or differentiated into subdomains that contain different kinds of proteins. These subdomains consist of the protein matrix, a proteinaceous crystalloid, phytin inclusions, and intravacuolar vesicles derived by autophagy. Most electron micrographs of partially filled seed PSVs show storage proteins aggregated and, in many cases, deposited adjacent to the inner surface of the tonoplast. Whether these aggregations represent low pH precipitation of the accumulated proteins or fixation-induced aggregation has been debated. E.M. Herman (unpublished data) has examined cryofixed maturing soybean cotyledons, observing that the storage proteins do not appear to be densely aggregated but are instead dispersed throughout the vacuole. This suggests that protein aggregation could be a fixation artifact.

The 7S and 11S seed storage proteins are members of large gene families and the most prominent PSV constituents (reviewed in Shewry et al., 1995). The 11S proteins are of ancient origin and are found in conifers as well as in dicotyledonous and monocotyledonous seeds (Higuchi and Fukazawa, 1987). In PSVs that possess crystalloids, the 7S proteins are in the peripheral matrix, whereas the 11S pro-

teins are the primary constituent of the crystalloid (Hara-Nishimura et al., 1985). In other species, the 7S and 11S proteins are uniformly codistributed within the PSV. Other proteins are sequestered with the storage proteins, and these may be in sufficient concentration to constitute auxiliary storage proteins.

The best examples of these auxiliary proteins are the seed lectins, which in some legumes can account for 10% or more of the total protein (reviewed in Etzler, 1985). Closely related to lectins are the α-amylase inhibitors, which defend against insects that feed on mature, dry seeds (reviewed in Chrispeels and Raikhel, 1991). Other PSV-localized defense proteins include Kunitz-type trypsin inhibitor (Horisberger and Volanthen, 1983) and P34 (Kalinski et al., 1992), a distantly related member of the papain superfamily that binds an elicitor secreted from *Pseudomonas* (Cheng et al., 1998). Defense proteins in dry seeds anticipate the possibility of insects feeding on the seed when it is unable to respond with an inducible reaction.

The accumulation of storage proteins within the vacuole is accompanied by additional processing that may serve to modify and prepare the proteins for dense packing. This processing includes modifications to both the polypeptide chain and glycan side chain, although not all PSV proteins are modified after deposition. The endoproteolytic cleavage of the 11S storage proteins into two chains linked by a disulfide bridge is evolutionarily conserved in seeds of conifers, monocots and dicots (Dickinson et al., 1989; reviewed in Nielsen et al., 1995). The conserved cleavage site is on the C-terminal side of an asparagine, and the responsible asparagine-specific endopeptidase, termed vacuolar processing enzyme (VPE), has been identified and its gene cloned (Hara-Nishimura et al., 1993a, 1995). Nielsen and colleagues have demonstrated that VPE-mediated cleavage at the conserved asparagine site is required to convert the oligomeric trimer formed in the ER to the mature vacuole-localized hexamer (11S) storage protein (Jung et al., 1998).

Other seed proteins mature similarly through processing by VPE. Among the more unusual forms of PSV-specific processing is the maturation of pro-concanavalin A. This protein is initially synthesized as a precursor (Herman and Shannon, 1985) that is glycosylated within a central short peptide that links the N- and C-terminal domains (Carrington et al., 1985). After cleavage at two asparagine sites, the glycosylated peptide is lost, the N- and C-terminal domains become reversed, and the former C-terminal domain is ligated to the former N-terminal domain, producing an intact and correctly folded protein (Bowles et al., 1986). PSV-localized glycosidases may remove exposed glycosyl residues from glycoproteins, in some cases removing sugar residues added to the glycan side chain prior to transport to the vacuole (Vitale and Chrispeels, 1984).

It may seem paradoxical that the vacuole serves as both the cellular protein storage compartment and lytic compartment. PSVs contain numerous enzymes capable of completely degrading macromolecules. Seed protein storage vacuoles contain diverse acid hydrolases, including glycosidases, phosphatases, phospholipase D, and nucleases (Nishimura and Beevers, 1978; Mettler and Beevers, 1979; Chappell et al., 1980; Herman and Chrispeels, 1980; Van der Wilden et al., 1980). Moreover, there is evidence that PSVs of maturing seeds do function as general lytic compartments that degrade materials derived from autophagy and endocytosis, as well as materials deposited as a result of progression through the endomembrane system.

Proteases present a particular problem to the storage function of the PSV. Seed storage proteins are specifically mobilized as a consequence of de novo synthesis of cysteine proteases after germination (Baumgartner and Chrispeels, 1977; Baumgartner et al., 1978), and storage proteins are good substrates for these enzymes (reviewed in Müntz, 1996). Recent studies, however, suggest that PSVs contain active proteases even while storage proteins accumulate. Expression of genes encoding foreign storage proteins, such as phaseolin and vicilin, in transgenic tobacco seeds usually results in stable protein accumulation in the same pattern as the intrinsic storage proteins. However, some storage proteins appear to be degraded in PSVs concurrently with the stable accumulation of intrinsic storage proteins. For example, a high-methionine mutant phaseolin (HiMet) is post-translationally unstable, although it is not so severely disrupted as to preclude its glycosylation and proper assembly (Hoffman et al., 1988; Lawrence et al., 1994). HiMet is stable while sequestered in the ER lumen, but it is rapidly degraded when it progresses to the PSV (Pueyo et al., 1995). This indicates that the conformation of the wild-type storage protein provides stability and resistance to colocalized PSV protease(s).

As subdivision and enlargement of the vacuole into PSVs occur, the tonoplast changes from a vegetative to a PSV-specific form. The tonoplasts of seed PSVs possess a differentiated polypeptide composition compared with their progenitors. The composition of isolated PSV tonoplasts includes up to 10 or 12 polypeptides (Mettler and Beevers, 1979; Pusztai et al., 1979; Weber et al., 1979). Of these, the first to be identified as a PSV-specific protein was the major 26-kD polypeptide of *Phaseolus vulgaris* (Mader and Chrispeels, 1984; Johnson et al., 1989). The gene encoding this protein proved to be a member of the membrane intrinsic protein family of pore proteins (Johnson et al., 1990), widely distributed in prokaryotic and eukaryotic cells (reviewed in Maurel, 1997; see also Chrispeels et al., 1999, in this issue). The 26-kD protein, α-TIP, has been shown to be an aquaporin, or water channel (Maurel et al., 1995), providing a functional explanation for the desiccation and hydration phases of the seed's life cycle. Accumulation of α-TIP occurs during late seed maturation, primarily after the subdivision of the vegetative vacuole is completed (Johnson et al., 1989, 1990; Melroy and Herman, 1991; Inoue et al., 1995). In vivo phosphorylation of α-TIP (Johnson et al., 1992) appears to control the water pore (Maurel et al., 1995).

Curiously, maturing PSV tonoplasts appear to be deficient

in the major tonoplast proton pump proteins, V-ATPase and pyrophosphatase, that function to acidify the vacuole (Maeshima et al., 1994; see also Sze et al., 1999, in this issue). How, or even if, the maturing PSV is acidified remains to be determined. Inoue et al. (1995) showed that pumpkin PSV tonoplasts possess two α-TIP isoforms and four other abundant proteins, including two glycoproteins of 27 and 32 kD and two other proteins of 73 and 80 kD. They determined that the 27- and 32-kD proteins are formed from a single precursor protein upon cleavage of the C-terminal side of an asparagine residue, which is presumably mediated by the VPE sequestered within the PSV. Although immunogold labeling assays showed the 27- and 32-kD proteins to be associated with the tonoplast, the sequence of the cDNA clone encoding these proteins does not indicate a transmembrane domain, thereby suggesting that the proteins are peripheral to the inner surface of the PSV tonoplast.

During germination and seedling growth, PSVs dedifferentiate, with the separate PSVs fusing to reform the vegetative vacuole. The fusion of up to a thousand PSVs into a single vacuole results in relatively little change in the total volume of the vacuole(s), but it does require a massive reduction in membrane surface area. The excess tonoplast is internalized, and PSV membrane proteins, including α-TIP and the 27- and 32-kD peripheral proteins, are degraded (Melroy and Herman, 1991; Inoue et al., 1995). In parallel, new tonoplast is contributed to the reforming vegetative vacuole by Golgi-derived secretion vesicles, which also carry enzymes that degrade the storage proteins. Tonoplast proteins such as pyrophosphatase, γ-TIP, and V-ATPase (Maeshima et al., 1994) are concomitantly delivered to the dedifferentiated central vacuole.

The regulation of water permeability through the PSV by aquaporins may be supported by the membrane structure. Specifically, PSV membranes contain high levels of sterols (Herman et al., 1984), which decrease membrane fluidity, so as to increase water accessibility to the hydrophilic portions of the membrane and decrease water accessibility to the hydrophobic membrane interior (Kusumi et al., 1986). Electron spin resonance has similarly established that the PSVs of germinating pumpkin cotyledons possess much more rigid membranes than do the tonoplasts of reformed vegetative vacuoles. Presumably, water transport could be regulated by the abundant α-TIP pores. This model would permit water transport during desiccation and rehydration to be regulated by phosphorylation of the α-TIP (Johnson et al., 1992; Maurel et al., 1995), which could in turn be regulated by an uncharacterized kinase and signal transduction system.

Protein Storage in Vegetative Tissues

Vegetative PSVs are structurally distinct from seed PSVs, although far less is known about vegetative PSV ontogeny. Vegetative cells store a wide variety of proteins (reviewed in Staswick, 1994) that are dissimilar to the seed-specific globulin and prolamin storage proteins. Seed lectins are abundant auxiliary storage proteins in some legume seeds, and isoforms of the seed lectins have been shown to be significant or primary storage proteins in the leaves and bark of tree legumes (Hankins et al., 1987, 1988). Other VSPs are related to acid phosphatase (DeWald et al., 1992; reviewed in Staswick, 1994) and to sporamin (Maeshima et al., 1985) and patatin from sweet potatoes and potatoes, respectively. In soybean, a lipoxygenase isoform appears to function together with the VSP as an auxiliary storage protein (Tranbarger et al., 1991).

All the VSPs described thus far are found in vacuoles and appear to be processed through the secretory system. As in seeds, VSPs appear to be deposited in vacuoles by a Golgi-mediated process (see, e.g., Klauer and Franceschi, 1997). Vegetative PSVs have been described in leaves, seed pods, stems, regreening cotyledons, bark, and storage tubers. In most instances, vegetative cells accumulate protein in pleiomorphic vacuole(s) that appear to be structurally distinct from the small spherical PSVs of seeds (see, e.g., Greenwood et al., 1986; Herman et al., 1988). Proteins deposited in vegetative PSVs may consist of sparse protein aggregates or densely packed protein entirely filling the vacuole. Vegetative protein storage may be restricted to specialized cells, such as the paraveinal mesophyll of soybean leaves (Franceschi and Giaquinta, 1983a, 1983b), or may be accumulated in the mesophyll cells that constitute most of the tissue bulk, as in tubers.

In contrast to seed storage protein accumulation, which is tightly regulated by developmental programming (reviewed in Thomas, 1993), VSP accumulation is regulated by complex factors that include both developmental and seasonal changes (see, e.g., Nsimba-Lubaki and Peumans, 1986; Wetzel et al., 1989) as well as source–sink (Staswick, 1989) and other environmental effects (van Cleve and Apel, 1993). Factors specific to vegetative cell storage vacuoles, as opposed to the PSVs of seeds, may have more to do with the parallel role of the former as a lytic and osmoregulatory organelle than with the specific requirements for protein storage and mobilization.

Among the key characteristics that differentiate the PSVs of the seed from vegetative vacuoles is the presence of different tonoplast proteins. Vegetative cells possess TIPs that may function in osmoregulation, whereas the desiccation-competent cells of seeds possess α-TIP, which probably functions in water efflux/influx during maturation and germination. Critical information is lacking on whether the tonoplast of vegetative cell PSVs becomes specialized during the course of protein accumulation or mobilization and whether it becomes further specialized during dormancy. Although there is little information on whether the tonoplast of vegetative cell PSVs is differentiated with respect to specialized polypeptides, Jauh et al. (1998) have presented evidence showing that the PSV of soybean leaves contains a specific TIP they have termed δ-TIP. In systems such as soybeans, isolating PSVs from one cell type of a complex

tissue may pose difficulties, but in systems such as tubers, where the storage cells are abundant, isolating the PSVs may be much easier. A simple approach may be to use quantitative electron microscope immunocytochemistry to study the process of vegetative protein accumulation and parallel changes in tonoplast structure.

PERSPECTIVES FOR THE FUTURE

There is much that remains to be learned about how plant cells store and utilize protein reserves. Most of the research to date has concentrated on a few model crop plant systems that may not represent the diversity of possible cellular mechanisms. The regulation of the subdivision of the vegetative vacuole in maturing seeds to form PSVs appears to be highly conserved, although mechanistic details are lacking. The mechanisms that control the stability of vegetative and seed storage proteins in the vacuole remain an interesting and uninvestigated area. What is the basis of storage protein resistance and sensitivity to vacuole proteases? Have storage proteins coevolved with vacuolar proteases so that pairs have been selected in which a storage protein is resistant to the cosequestered proteases during the accumulation phase and susceptible to different newly synthesized proteases during the mobilization phase? Autophagy of PBs is a significant mechanism for protein accumulation in the endosperm of some cereals, and this process can be induced in transgenic tobacco plants. If autophagy of PBs is selective, tobacco may provide a good model system to elucidate the mechanism of induced autophagy. Investigations into the role of reserve proteins in allowing plants to cope with seasonal changes and environmental stress are also likely to yield many new observations.

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